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# Cellular and subcellular localization of enzymes of arginine metabolism in rat kidney

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Rat kidneys extract citrulline derived from the intestinal metabolism of glutamine and convert it stoichiometrically into arginine. This pathway constitutes the major endogenous source of arginine. We investigated the localization of enzymes of arginine synthesis, argininosuccinate synthase and lyase, and of breakdown, arginase and ornithine aminotransferase, in five regions of rat kidney, in cortical tubule fractions and in subcellular fractions of cortex. Argininosuccinate synthase and lyase were found almost exculsively in cortex. Arginase and ornithine aminotransferase were found in inner cortex and outer medulla. Since cortical tissue primarily consists of proximal convoluted and straight tubules, distal tubules and glomeruli, we prepared cortical tubule fragments by collagenase digestion of cortices and fractionated them on a Percoll gradient. Argininosuccinate synthase and lyase were found to be markedly enriched in proximal convoluted tubules, whereas less than 10% of arginase and ornithine aminotransferase, were recovered in this fraction. Arginine production from citrulline was also enriched in proximal convoluted tubules. Subcellular fractionation of kidney cortex revealed that argininosuccinate synthase and lyase are cytosolic. We therefore conclude that arginine synthesis occurs in the cytoplasm of the cells of the proximal convoluted tubule.

## INTRODUCTION

Renal arginine synthesis has been known for many years (Borsook & Dubnoff, 1941; Ratner & Petrack, 1953), but its significance has only recently become apparent. Studies by Featherston *et al.* (1973) showed that the kidneys are the major biosynthetic source of circulating arginine. Citrulline, derived from intestinal metabolism of glutamine (Windmueller & Spaeth, 1981), can be quantitatively converted into arginine and released to the circulation (Dhanakoti *et al.*, 1990). The importance of this pathway to the body's arginine status is well illustrated by the studies by Hoogenraad *et al.* (1985), who showed that arginine becomes a dietary essential amino acid when intestinal citrulline synthesis is inhibited.

The enzymes of arginine synthesis from citrulline are argininosuccinate synthase (L-citrulline:L-aspartate ligase, EC 6.3.4.5) and argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1). Their location in the kidney is not well characterized. Szepesi *et al.* (1970) reported that arginine synthase activity (measured as argininosuccinate synthase plus argininosuccinate lyase) was predominantly found within the renal medulla. Morris *et al.* (1989) have more recently shown that the mRNAs for argininosuccinate synthase and argininosuccinate lyase are localized in the renal cortex. Thus these two reports are in conflict. The kidney also contains enzymes that can catabolize arginine, specifically arginase (L-arginine amidinohydrolase, EC 3.5.3.1) and ornithine aminotransferase (EC 2.6.1.13). Szepesi *et al.* (1970) found arginase in both cortex and medulla. Levillain *et al.* (1989) have shown, by micro-dissecting the nephron, that arginase is almost exclusively localized in the proximal straight tubule. Skrzypek-Osiecka & Porembska (1983) have reported that arginase is predominantly found in the mitochondrial matrix of rat kidney. Ornithine aminotransferase has been found in the renal mitochondrial fraction (Peraino & Pitot, 1963), but its cellular location has not been established.

Because of the importance of renal arginine synthesis, we consider that definitive information on the location of the enzymes of its synthesis is required. We report here that these enzymes are exclusively present in the cytoplasm of the cells of the proximal convoluted tubule and that the catabolic enzymes are enriched in other kidney regions.

## MATERIALS AND METHODS

### Animals

In all experiments, male Sprague–Dawley rats (Charles River, Montreal, Canada) weighing 350–450 g were used. They were allowed water and Purina rat chow *ad libitum*.

### Preparation of homogenates from different kidney regions

Rats were killed by cervical dislocation and the kidneys were rapidly removed, decapsulated, placed in ice-cold homogenization medium (0.33 M-sucrose/5 mM-Hepes/1 mM-EGTA, pH 7.4, with 1 mM-dithiothreitol added just before use). The kidneys were bisected and further cut into cones, which clearly displayed the cortex, medulla and papilla. Each cone was dissected into five regions, starting from the outside: outer cortex, inner cortex, outer medulla, inner medulla and papilla (Lowry *et al.*, 1986). Tissue regions from two or three animals were pooled, and 10% (w/v) homogenates were prepared in the above homogenization medium to which 0.5% (v/v) Triton X-100 (final concn.) was added. The tissue was homogenized with a Polytron (Brinkman Instruments, Toronto, Canada) for 10–20 s at setting 20. Samples of these 10% homogenates were used for various enzyme and protein assays.

### Preparation and fractionation of kidney cortical tubules

Tubules were prepared from the kidney cortex of rats by collagenase digestion by the method of Guder *et al.* (1971), and viability was assessed by measuring the leakage of lactate dehydrogenase as described by Dhanakoti *et al.* (1990). The tubules were then suspended in 50% (v/v) Percoll (Pharmacia Fine Chemicals, Montreal, Canada) and fractionated by the method of Vinay *et al.* (1981). The fractionation of whole cortical-tubule suspension ($F_t$) yielded four fractions ($F_1$–$F_4$). These fractions were washed three times by centrifugation and resuspension in ice-cold Krebs–Henseleit (1932) saline (pH 7.4). The final pellets of the fractions and $F_t$ were resuspended in

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homogenization medium (approx. 6 times the tissue wet weight) containing 0.5% Triton X-100 (final concn.) and homogenized as described above. Samples of the homogenized fractions were employed for protein and enzyme assays.

### Arginine production in kidney cortical-tubule fractions

Kidney cortical tubules were prepared and fractionated as described above. Tubule fractions ($F_1$–$F_4$) and $F_t$ were washed as described above and resuspended in ice-cold Krebs–Henseleit saline (pH 7.4). They were incubated (about 1 mg dry wt.) with substrates (Dhanakoti *et al.*, 1990), in a total volume of 2 ml of Krebs–Henseleit saline (pH 7.4) equilibrated with $O_2/CO_2$ (19:1) for 30 min at 37 °C. Incubations were terminated with 0.2 ml of 30% (w/v) $HClO_4$. After removal of the protein by centrifugation, a portion of the $HClO_4$ extract was filtered through a 0.45 $\mu$m-pore filter (Millipore), and arginine was determined by a modification of the h.p.l.c. procedure developed by Seiler *et al.* (1985), as described by Dhanakoti *et al.* (1990).

### Homogenization and fractionation of kidney cortex

This was done essentially by the method of de Duve *et al.* (1955), with slight modifications as employed by Kalra & Brosnan (1974). Triton X-100 (final concn. 0.5%) was added to the fractions and to a portion of the initial homogenate, and these were then re-homogenized with a Polytron to ensure disruption of membranes. These homogenates were then used for protein and enzyme assays.

### Enzyme assays

All enzyme assays were carried out on the same day on freshly isolated tissue, tubule fractions and subcellular fractions. All enzyme assays were demonstrated to be linear with time and with protein concentration. The marker enzymes assayed were: $\gamma$-glutamyltranspeptidase (Tate & Meister, 1974), alkaline phosphatase (Scholer & Edelman, 1979), phosphoenolpyruvate carboxykinase (Seubert & Huth, 1965), $\beta$-glucuronidase (Gianetto & de Duve, 1955) (measured at 37 °C); succinate- and NADPH–cytochrome *c* reductase (Sottocassa *et al.*, 1967), lactate dehydrogenase (Morrison *et al.*, 1966) and hexokinase (Vinuela *et al.*, 1963), measured at room temperature.

We assayed four enzymes of arginine metabolism: argininosuccinate synthase, argininosuccinate lyase, arginase and orthinine aminotransferase. Argininosuccinate synthase was assayed essentially by the method of Nuzum & Snodgrass (1976), except that [$^{14}$C]urea formed from [*ureido*-$^{14}$C]citrulline was measured as $^{14}CO_2$ released after treatment with urease. The incubation mixture (pH 7.5) (containing about 0.25 mg of protein in a total volume of 1.0 ml) contained the following at the indicated final concentrations: 50 mM-$KH_2PO_4$, 5 mM-potassium aspartate, 5 mM-[*ureido*-$^{14}$C] citrulline, (370 kBq/mmol), 2 mM-ATP, 2 mM-$MgSO_4$, 2 mM-phosphoenolpyruvate, 3 units of pyruvate kinase (rabbit muscle; type II, Sigma), 0.8 units of argininosuccinate lyase (bovine liver; type IV, Sigma), 5 units of arginase (bovine liver; type IV, Sigma), 14 units of urease (Jack bean; type VI, Sigma). The incubations were carried out in flasks fitted with centre wells containing 0.2 ml of NCS (Amersham) at 37 °C for 0 and 30 min and terminated by injecting, through the rubber cap, 0.2 ml of 30% $HClO_4$. After 60 min $^{14}CO_2$ collected in centre wells was counted for radioactivity in a scintillation counter. Argininosuccinate lyase was assayed by the method of Nuzum & Snodgrass (1976), with slight modifications. Briefly, the incubation mixture (pH 7.0) contained 20 mM-argininosuccinate, 129 mM-$NaH_2PO_4$, 64.5 mM-EDTA and 1 mg of protein in a total volume of 1.0 ml. Incubations were terminated with 0.5 ml of 20% (w/v) trichloroacetic acid after 0 and 15 min at 37 °C. Samples of supernatants, after removal of protein by centrifugation, were used for arginine determination by the Sakaguchi colour reaction as described by Van Pilsum *et al.* (1956).

Arginase was assayed with 0.25 mg of protein at 37 °C for 10 min by the method of Herzfeld & Raper (1976), after heat activation of the various homogenates at 55 °C for 5 min in the presence of 50 mM-$MnCl_2$. Urea formed in the arginase assay was quantified by the procedure of Geyer & Dabich (1971). Ornithine aminotransferase activity was determined with 1.0 mg of protein (15 min at 37 °C) by measuring the formation of glutamic $\gamma$-semialdehyde by the procedure of Peraino & Pitot (1963) as modified by Pegg *et al.* (1970).

Protein was measured by the biuret method (Gornall *et al.*, 1949), after solubilization with deoxycholate (Jacobs *et al.*, 1956), and with BSA as standard. DNA was extracted by the method of Schneider (1945), and was determined with diphenylamine reagent (Burton, 1956), with calf thymus DNA as standard.

### Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), except [*ureido*-$^{14}$C]citrulline (1.57 GBq/mmol), which was obtained from New England Nuclear (Lachine, Quebec, Canada) and was purified by the method of Hurwitz & Kretchmer (1986) before use.

## RESULTS AND DISCUSSION

Our strategy for establishing the cellular and subcellular localization of the renal enzymes of arginine metabolism relied on the use of defined marker enzymes. This is especially important with respect to the kidney, where such terms as inner cortex, outer medulla etc. seem to mean different things in different laboratories. The discrepancies in the location of these enzymes mentioned in the Introduction may relate to this problem. Accordingly, we use the terminology recommended by Kriz & Bankir (1988) for the different parts of the nephron. We also use, as markers, enzymes whose location has been unambiguously established. We have employed two enzymes known to be localized in the brush-border membrane of the proximal tubule. Alkaline phosphatase is found throughout the length of the proximal tubule ($S_1$, $S_2$ and $S_3$ segments) (Schmidt & Dubach, 1971), whereas $\gamma$-glutamyltranspeptidase is highly enriched in the straight segment ($S_3$) of this tubule (Heinle *et al.*, 1977). Phosphoenolpyruvate carboxykinase is found throughout the proximal tubule, with somewhat higher activities being found in the early convoluted ($S_1$, $S_2$) portions (Guder & Schmidt, 1974). Hexokinase is essentially absent from the proximal tubule, but is present in all later tubular segments (Schmidt *et al.*, 1975). The principal utility of hexokinase in the present context is that very low activities are diagnostic of proximal tubules. One of the enzymes of arginine utilization, arginase, has been established, by micro-dissection studies, to be highly enriched in the proximal straight tubule ($S_3$) (Levillain *et al.*, 1989), and thus it should closely follow the distribution of $\gamma$-glutamyltranspeptidase.

### Distribution of enzymes of arginine metabolism in different kidney regions

The specific and total activities for the marker enzymes and enzymes of arginine metabolism in different zones of rat kidney are presented in Table 1. The protein content in cortex (outer and inner), medulla (outer and inner) and papilla accounted for about 83%, 15% and 2.6% respectively.

The specific activities of the brush-border enzymes $\gamma$-glutamyltranspeptidase and alkaline phosphatase were highest in the inner cortex, but high activities were also evident in the outer cortex and medulla. About 85% of $\gamma$-glutamyltranspeptidase

**Table 1. Distribution of enzymes of arginine metabolism in different regions of the rat kidney**

The enzyme activities were assayed as described in the Materials and methods section. All specific activities are expressed as μmol/min per g of protein. The total activity of each enzyme refers to the activity in μmol/min in each region of a 1 g (wet wt.) kidney. Values are means ± S.D. for three experiments. Values in parentheses represent percentage recovery.

| | Kidney region... | Cortex (outer) | Cortex (inner) | Medulla (outer) | Medulla (inner) | Papilla |
|---|---|---|---|---|---|---|
| Protein | (mg/g wet wt. of whole kidney) | 90.4±9.4 (53.9) | 49.4±5.9 (29.4) | 13.7±2.0 (8.2) | 10.1±0.5 (6.0) | 4.4±2.0 (2.6) |
| γ-Glutamyl-transpeptidase | Specific activity | 2992±894 | 5566±338 | 4638±1936 | 3735±1653 | 274±39 |
| | Total actvity | 267±69 (41.2) | 277±50 (43.3) | 65.7±36.7 (9.7) | 38.1±18.8 (5.6) | 1.25±0.69 (0.2) |
| Alkaline phosphatase | Specific activity | 72.6±18.8 | 102.4±7.6 | 91.3±24.4 | 77.1±4.1 | 7.4±2.1 |
| | Total activity | 6.48±1.40 (48.1) | 5.03±0.33 (37.9) | 1.28±0.53 (9.3) | 0.61±0.20 (4.5) | 0.03±0.01 (0.2) |
| Phosphoenolpyruvate carboxykinase | Specific activity | 67.7±9.9 | 44.1±1.1 | 12.6±6.3 | 12.0±5.3 | 3.9±0.8 |
| | Total activity | 6.06±0.35 (70.9) | 2.17±0.21 (25.4) | 0.18±0.11 (2.1) | 0.12±0.06 (1.4) | 0.02±0.005 (0.2) |
| Hexokinase | Specific activity | 10.4±3.2 | 14.4±4.1 | 29.5±12.4 | 35.9±11.7 | 24.1±13.3 |
| | Total activity | 0.94±0.30 (37.6) | 0.69±0.13 (28.7) | 0.39±0.12 (15.7) | 0.36±0.11 (14.4) | 0.09±0.03 (3.6) |
| Argininosuccinate synthase | Specific activity | 7.5±1.9 | 3.8±1.1 | 3.8±0.5 | 2.7±1.5 | 2.7±0.5 |
| | Total activity | 0.69±0.25 (70.6) | 0.18±0.05 (19.9) | 0.05±0.01 (5.6) | 0.03±0.01 (2.7) | 0.011±0.005 (1.2) |
| Argininosuccinate lyase | Specific activity | 3.9±1.6 | 2.3±1.4 | 1.0±0.5 | 1.1±0.8 | 0.7±0.7 |
| | Total activity | 0.34±0.12 (73.0) | 0.11±0.07 (21.5) | 0.014±0.007 (2.9) | 0.011±0.008 (2.1) | 0.003±0.001 (0.5) |
| Arginase | Specific activity | 124±26.5 | 289±43 | 348±22 | 267±125.5 | 201±24.5 |
| | Total activity | 11.08±1.41 (33.0) | 14.25±2.35 (42.3) | 4.78±0.84 (14.2) | 2.73±1.42 (7.9) | 0.89±0.43 (2.7) |
| Ornithine aminotransferase | Specific activity | 18.2±1.7 | 34.1±4.6 | 33.7±5.2 | 20.8±3.4 | 5.5±1.7 |
| | Total activity | 1.64±0.09 (41.1) | 1.68±0.30 (41.7) | 0.47±0.13 (11.5) | 0.21±0.04 (5.2) | 0.02±0.006 (0.5) |

and alkaline phosphatase were found in the cortex, with 41 and 48% respectively being recovered in outer cortex alone. These results are in agreement with those reported by Lowry *et al.* (1985, 1986). Phosphoenolpyruvate carboxykinase was essentially restricted to the cortex, and was predominantly found in the outer cortex, which also had the highest specific activity. The distribution of argininosuccinate synthase and argininosuccinate lyase was very similar to that of phosphoenolpyruvate carboxykinase, in that the cortex contained about 90–95% of each enzyme, with the greatest amount and the highest specific activity being found in the outer cortex. The specific activity of hexokinase was lower in outer cortex than in medulla and papilla. About 38% of hexokinase was found in outer cortex. From previous studies (Vinay *et al.*, 1981) we know that cortical hexokinase is associated with distal tubules. Arginase and ornithine aminotransferase were similarly distributed between the regions. In each case most of the enzyme was found in cortex, with the highest enrichments in the inner cortex and outer medulla.

The predominant location of argininosuccinate synthase and argininosuccinate lyase and of arginase and ornithine aminotransferase in the kidney cortex prompted us further to determine the abundance of these enzymes in tubule fractions from the cortex. Cortex is a heterogeneous tissue, and fractionation of the cortical tubules on a Percoll gradient permits substantial purification of various elements.

### Distribution of enzymes of arginine metabolism in kidney cortical-tubule fractions

The data are shown in Table 2. The total recovery of protein in tubule fractions $F_1$–$F_4$ from the original cortical-tubule suspension ($F_t$) was about 60–63% and slightly lower than that obtained (70%) by Vinay *et al.* (1981). Isolated cells and cell debris sediment between the bands and are lost. Some further losses occur during the washing of the tubule fractions to remove Percoll (Vinay *et al.*, 1981). All parts of the tubule appear to be equally affected, as there are comparable recoveries of the marker enzymes for the various fractions. The specific activity of γ-glutamyltranspeptidase was higher in $F_1$ than in $F_4$, and that of alkaline phosphatase was higher in $F_4$ than in $F_1$ fractions. The total recoveries for γ-glutamyltranspeptidase and alkaline phosphatase were 58 and 73% respectively. A 1.5-fold increase in specific activity was observed for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (marker for proximal convoluted and straight tubules) in $F_4$ fraction; the ratio $F_4/F_1$ was 1.88 and the recovery of the enzyme was about 72%. On the other hand, the specific activity of the glycolytic enzyme hexokinase (marker for distal tubules) was about 1.7-fold higher in $F_1$, the ratio $F_4/F_1$ was 0.23 and the total recovery was about 67%. These results are in agreement with those of Vinay *et al.* (1981). From the distribution of these marker enzymes we can conclude that $F_4$ consists primarily of proximal convoluted tubules ($S_1$ and $S_2$). This conclusion can be sustained from the enrichment in $F_4$ of both phosphoenolpyruvate carboxykinase and alkaline phosphatase (both present throughout the proximal tubule), together with the low activities in $F_4$ of the two enzymes (γ-glutamyltranspeptidase and arginase) known to be found in the proximal straight ($S_3$) segments. The very low activity of hexokinase in $F_4$ attests to the minimal presence of distal tubules in this fraction. The enrichment of hexokinase, arginase and γ-glutamyltranspeptidase in $F_1$ indicates that this fraction contains

**Table 2. Distribution of enzymes of arginine metabolism in rat kidney cortical-tubule fractions**

Kidney cortical tubules were prepared by collagenase digestion and then fractionated on Percoll gradients. $F_t$ is the fraction before fractionation. $F_1$, $F_2$, $F_3$ and $F_4$ are the fractions obtained after fractionation. The enzyme activities were assayed as described in the Materials and methods section. All specific activities are expressed as $\mu$mol/min per g of protein. Total activity is expressed as $\mu$mol/min per fraction. Values are means ± S.D. for three experiments. Values in parentheses represent percentage recovery.

| | Tubule fraction... | $F_t$ | $F_1$ | $F_2$ | $F_3$ | $F_4$ | Total recovery (%) | $F_4/F_1$ |
|---|---|---|---|---|---|---|---|---|
| Protein | (mg) | 83.9±24.6 (100) | 14.4±2.1 (17.1) | 11.3±2.9 (13.5) | 9.0±2.3 (12.1) | 14.6±4.6 (17.4) | 60 | |
| γ-Glutamyl-transpeptidase | Specific activity | 2469±928 | 3167±692 | 3311±1884 | 1586±276 | 1319±458 | 58 | 0.42 |
| | Total activity | 191.9±17.5 (100) | 44.6±3.5 (23.3) | 34.3±10.7 (18.1) | 14.0±2.6 (7.2) | 17.8±1.0 (9.4) | | |
| Alkaline phosphatase | Specific activity | 252.4±94.9 | 230.2±11.8 | 295.2±89.3 | 300±93.3 | 370.1±81.8 | 73 | 1.61 |
| | Total activity | 19.8±3.4 (100) | 3.31±0.50 (16.9) | 3.20±0.36 (16.3) | 2.56±0.20 (13.1) | 5.14±0.82 (26.5) | | |
| Phosphoenolpyruvate carboxylinase | Specific activity | 47.5±11.8 | 37.4±10.2 | 61.8±14.3 | 58.8±7.9 | 70.2±5.5 | 72 | 1.88 |
| | Total activity | 3.88±1.21 (100) | 0.52±0.1 (14.0) | 0.67±0.07 (18.0) | 0.53±0.14 (13.7) | 1.02±0.33 (26.4) | | |
| Hexokinase | Specific activity | 9.0±0.06 | 15.2±3.2 | 14.0±3.6 | 8.4±2.5 | 3.5±0.9 | 67 | 0.23 |
| | Total activity | 0.76±0.22 (100) | 0.22±0.08 (29.4) | 0.16±0.06 (21.2) | 0.08±0.03 (9.8) | 0.05±0.02 (6.7) | | |
| Argininosuccinate synthase | Specific activity | 3.20±0.74 | 3.97±1.09 | 7.9±4.84 | 7.42±5.41 | 12.34±7.19 | 143 | 3.11 |
| | Total activity | 0.256±0.026 (100) | 0.056±0.006 (2.21) | 0.086±0.05 (34.1) | 0.058±0.03 (23.5) | 0.158±0.05 (62.8) | | |
| Argininosuccinate lyase | Specific activity | 2.36±1.94 | 1.26±0.56 | 3.88±2.82 | 9.02±8.0 | 13.62±9.60 | 175 | 10.81 |
| | Total activity | 0.17±0.1 (100) | 0.019±0.01 (14.4) | 0.041±0.02 (23.3) | 0.069±0.05 (35.4) | 0.178±0.12 (101.7) | | |
| Arginase | Specific activity | 71.2±38.5 | 132.9±65.0 | 79.8±31.9 | 29.7±7.3 | 12.0±4.0 | 66 | 0.09 |
| | Total activity | 5.96±4.0 (100) | 1.93±1.0 (34.6) | 0.90±0.46 (21.6) | 0.27±0.11 (5.7) | 0.17±0.08 (3.8) | | |
| Ornithine amino-tranferase | Specific activity | 9.3±3.9 | 20.7±5.5 | 13.2±4.8 | 11.1±7.9 | 3.9±2.6 | 84 | 0.19 |
| | Total activity | 0.71±0.14 (100) | 0.30±0.08 (41.9) | 0.15±0.04 (22.0) | 0.09±0.04 (12.6) | 0.05±0.02 (7.5) | | |

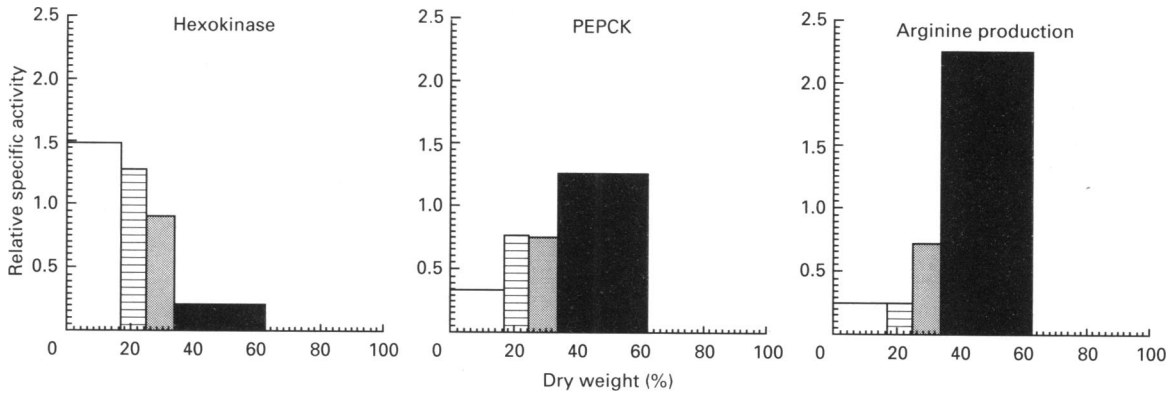


**Fig. 1. Arginine production in kidney cortical tubule fractions**

The Figure shows a plot (de Duve *et al.*, 1955) of mean values obtained from three separate experiments. Kidney cortical tubules were prepared by collagenase digestion and then fractionated on a Percoll gradient. $F_1$ (□), $F_2$ (▤), $F_3$ (▨) and $F_4$ (■) are the fractions obtained. The relative specific activity is plotted against dry weight (%), which is the percentage recovery of the dry weight of the original tubule suspension. The relative specific activity is calculated as the specific activity in the fraction divided by that in the original tubule suspension. The specific activities of hexokinase, phosphoenolpyruvate carboxykinase (PEPCK) and arginine production in the original tubule suspension were 8.6, 32.4 and 1.68 $\mu$mol/min per g dry wt. respectively.

proximal straight and distal tubules. We did not succeed in further subfractionating $F_1$ into distal tubules and proximal straight tubules.

Both the enzymes of arginine synthesis (argininosuccinate synthase and lyase) were found predominantly in $F_4$, so we conclude that these two enzymes primarily occur in the proximal convoluted tubule. This is consistent with their occurrence in the outer cortex (Table 1).

It should be noted that the total recoveries of argininosuccinate synthase and lyase were 143 and 175% respectively. These

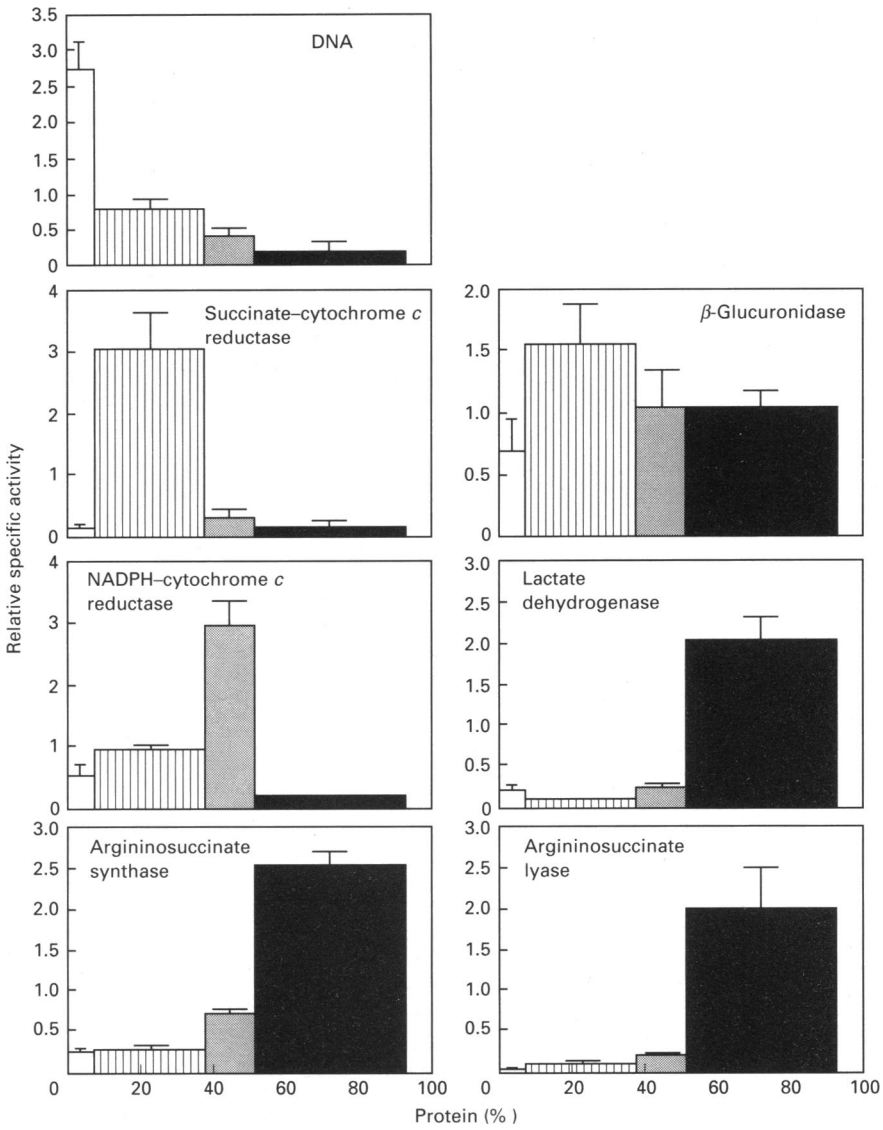


**Fig. 2. Subcellular localization of enzymes of arginine synthesis in rat kidney cortex**

The Figure depicts a plot (de Duve *et al.*, 1955) of relative specific activity versus protein (%). Kidney cortex homogenate was fractionated and the enzymes, protein and DNA were assayed as described in the Materials and methods section. Results are presented as means ± S.D. ($n = 3$). Protein (%) is the percentage recovery of protein of the cortex homogenate. The relative specific activity is calculated as the specific activity in the fraction divided by that in the cortex homogenate. The DNA content in the homogenate was 23.2 ± 2.2 μg/mg of protein. The specific activities (μmol/min per g of protein) of the marker enzymes in the homogenate were 29.3 ± 4.8 (succinate–cytochrome *c* reductase), 1.56 ± 0.55 (β-glucuronidase), 1.79 ± 0.60 (NADPH–cytochrome *c* reductase) and 1344 ± 187 (lactate dehydrogenase). The specific activities of argininosuccinate synthase and argininosuccinate lyase in the cortex homogenate were 9.44 ± 0.34 and 4.16 ± 0.61 respectively. Fractions: □, nuclear; ▥, mitochondrial + lysosomal; ▨, microsomal; ■, cytosolic.

results raised the possibility that there might be an inhibitor(s) of arginine synthesis in the original tubule suspension. If there were such an inhibitor, then the enrichment of those two enzymes in $F_4$ could be attributed to the loss of an inhibitor from this fraction. On the other hand, if the fractionation procedure has resulted in the complete loss of the putative inhibitor, then the results would be reliable. To address this question, we separated cortical tubules and then recombined them to give a reconstituted fraction. We then determined argininosuccinate synthase and argininosuccinate lyase activities in this reconstituted fraction and compared them with the activity in the original tubule suspension. Again, we observed an increase in the activities of the two enzymes in the sum of the four fractions. However, the total recovery of each enzyme in the reconstituted fraction was not different from that in the sum of the fractions. Since the reconstitution experiments showed that fractionation resulted in the disappearance of all of the putative inhibitor(s), we can be confident that the enrichment of the enzymes of arginine synthesis in $F_4$ is not an artifact caused by a retention of inhibitor in one or the other fractions.

Ornithine aminotransferase is enriched in $F_1$, but we are unable to determine whether it is localized in distal or in proximal straight tubules. It does follow the distribution of arginase closely, and it would be reasonable to find it in the proximal straight tubule together with this enzyme, since it could metabolize any ornithine produced by the action of arginase. However, definitive experiments need to be carried out to establish unambiguously the nephron location of ornithine aminotransferase. On the other hand, it is clear that the enzymes of arginine synthesis and of arginine degradation are present in

different cells, as is apparent from the very low arginase activity in $F_4$. Thus newly synthesized arginine is not immediately subjected to catabolism, but may be released from extra-renal utilization. In this connection we have demonstrated a mole-for-mole relationship between renal citrulline uptake and arginine release *in vivo* (Dhanakoti *et al.*, 1990).

### Arginine production in kidney cortical-tubule fractions

Since we localized argininosuccinate synthase and argininosuccinate lyase in the $F_4$ fraction (proximal convoluted tubules), we proceeded to determine whether the metabolic production of arginine from citrulline would also be enriched here. Arginine production in different tubule fractions followed closely the distribution of phosphoenolpyruvate carboxykinase (Fig. 1). The recoveries for hexokinase, phosphoenolpyruvate carboxykinase and arginine production were about 50, 75 and 102% respectively. The rates of arginine production were 0.57 and 5.36 $\mu$mol/min per g of protein in fractions $F_1$ and $F_4$ ($F_4/F_1 = 9.4$) respectively, compared with 2.36 $\mu$mol/min per g of protein in the original cortical tubule suspension. It is possible that newly synthesized arginine could be catabolized by tissue arginase and thus result in an underestimation of the rate of synthesis. We performed a number of experiments to determine the extent of this. Tubules were incubated with [$^{14}$C]citrulline, and incorporation of $^{14}$C into both arginine and urea was assessed as described by Levillain *et al.* (1990). Urea production was always less than 7% of the rate of arginine synthesis (results not shown). These experiments confirm our enzyme-localization data that arginine synthesis occurs in proximal convoluted tubules, and are in agreement with the work of Levillain *et al.* (1990), who studied micro-dissected nephron segments. One noticeable difference between arginine synthesis and phosphoenolpyruvate carboxykinase was that arginine synthesis was even more enriched in $F_4$. This suggests that the putative inhibitor that decreased the activities of the enzymes of arginine synthesis in homogenates (Table 2) was also active in inhibiting arginine synthesis in intact cells. Again, this apparent inhibition was lost upon fractionation.

### Subcellular localization of enzymes of arginine synthesis in cortex

The data presented in Fig. 2 clearly demonstrate that argininosuccinate synthase and argininosuccinate lyase are located in the cytosolic fraction, as they followed the distribution of the cytosolic enzyme lactate dehydrogenase. The specific activities in the cytosolic fraction and the total recoveries for argininosuccinate synthase and argininosuccinate lyase were 23.6 and 8.04 $\mu$mol/min per g of protein and 122 and 86% respectively. Once again, the higher recovery observed for argininosuccinate synthase could be attributed to the presence of the putative inhibitor which was lost during the fractionation procedure. The specific activities and the total recoveries for the marker enzymes succinate–cytochrome *c* reductase, $\beta$-glucuronidase, NADPH–cytochrome *c* reductase, lactate dehydrogenase, DNA and protein were in agreement with those reported by de Duve *et al.* (1955) and Kalra & Brosnan (1974). In a previous report, in which no marker enzymes were employed, Kato *et al.* (1976) found argininosuccinate synthase and argininosuccinate lyase in soluble fractions. These authors also observed an increase in total enzyme activity after fractionation. This observation is consistent with ours in indicating the presence of an inhibitor of these enzymes.

### Concluding remarks

The results of our study clearly establish that arginine synthesis occurs in the cytosol of the cells of the proximal tubule in the rat kidney. We have previously shown that urine contains virtually no citrulline, so that essentially all of the filtered citrulline is re-absorbed (Dhanakoti *et al.*, 1990). Since the proximal tubule is the predominant site of renal amino acid re-absorption (Silbernagl, 1988), the localization of the enzymes of arginine synthesis in these cells would ensure that filtered citrulline would be available for arginine synthesis.

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## REFERENCES

Borsook, H. & Dubnoff, J. W. (1941) J. Biol. Chem. **141**, 717–738
Burton, K. (1956) Biochem. J. **62**, 315–323
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Applemans, F. (1955) Biochem. J. **60**, 604–617
Dhanakoti, S. N., Brosnan, J. T., Herzberg, G. R. & Brosnan, M. E. (1990) Am. J. Physiol. **259**, E437–E442
Featherston, W. R., Rogers, Q. R. & Freedland, R. A. (1973) Am. J. Physiol. **224**, 127–129
Geyer, J. W. & Dabich, D. (1971) Anal. Biochem. **39**, 412–417
Gianetto, R. & de Duve, C. (1955) Biochem. J. **59**, 433–438
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) J. Biol. Chem. **223**, 147–156
Guder, W. G. & Schmidt, U. (1974) Hoppe-Seyler's Z. Physiol. Chem. **355**, 273–278
Guder, W. G., Weisner, W. & Stukowski, B. (1971) Hoppe-Seyler's Z. Physiol. Chem. **352**, 1319–1328
Heinle, H., Wendel, A. & Schmidt, U. (1977) FEBS Lett. **73**, 220–224
Herzfeld, A. & Raper, S. M. (1976) Biochem. J. **153**, 469–478
Hoogenraad, N., Totino, N., Elmer, H., Wraight, C., Alewood, P. & Johns, R. B. (1985) Am. J. Physiol. **249**, G792–G799
Hurwitz, R. & Kretchmer, N. (1986) Am. J. Physiol. **251**, G103–G110
Jacobs, E. E., Jacob, J., Sanadi, D. R. & Bradley, L. B. (1956) J. Biol. Chem. **223**, 147–156
Kalra, J. & Brosnan, J. T. (1974) J. Biol. Chem. **249**, 3255–3260
Kato, H., Oyamada, I., Funahashi, M. & Nakagawa, H. (1976) J. Biochem. (Tokyo) **79**, 945–953
Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–66
Kriz, W. & Bankir, L. (1988) Am. J. Physiol. **254**, F1–F8
Levillain, O., Hus-Citharel, A., Morel, F. & Bankir, L. (1989) Renal Physiol. Biochem. **12**, 302–312
Levillain, O., Hus-Citharel, A., Morel, F. & Bankir, L. (1990) Am. J. Physiol. **259**, F916–F923
Lowry, M., Hall, D. E. & Brosnan, J. T. (1985) Metab. Clin. Exp. **34**, 955–961
Lowry, M., Hall, D. E. & Brosnan, J. T. (1986) Am. J. Physiol. **250**, F649–F658
Morris, S. M., Jr., Moncman, C. L., Holub, J. S. & Hod, Y. (1989) Arch. Biochem. Biophys. **273**, 230–237
Morrison, G. R., Brock, F. E., Sobral, D. T. & Shank, R. E. (1966) Arch. Biochem. Biophys. **114**, 494–501
Nuzum, C. T. & Snodgrass, P. J. (1976) in The Urea Cycle (Grisolia, S., Baguena, R. & Mayor, F., eds.), pp. 325–349, John Wiley and Sons, New York
Pegg, A. E., Lockwood, D. H. & Williams-Ashman, H. G. (1970) Biochem. J. **117**, 17–31
Peraino, C. & Pitot, H. C. (1963) Biochim. Biophys. Acta **73**, 222–231
Ratner, S. & Petrack, B. (1953) J. Biol. Chem. **200**, 175–185
Schmidt, U. & Dubach, U. C. (1971) Progr. Histochem. Cytochem. **2**, 185–298
Schmidt, U., Marosvari, J. & Dubach, U. C. (1975) FEBS Lett. **53**, 26–28
Schneider, W. C. (1945) J. Biol. Chem. **161**, 293–303
Scholer, D. W. & Edelman, I. S. (1979) Am. J. Physiol. **237**, F350–F359
Seiler, N., Sarhan, S. & Knodgen, B. (1985) Int. J. Dev. Neurosci. **3**, 317–322

Seubert, W. & Huth, W. (1965) Biochem. Z. **343**, 176–191
Silbernagl, S. (1988) Physiol. Rev. **68**, 911–1007
Skrzypek-Osiecka, I. & Porembska, Z. (1983) Acta Biochim. Pol. **30**, 83–92
Sottocassa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) J. Cell Biol. **32**, 415–438
Szepesi, B., Avery, E. H. & Freedland, R. A. (1970) Am. J. Physiol. **219**, 1627–1631
Tate, S. S. & Meister, A. (1974) J. Biol. Chem. **249**, 7593–7602
Van Pilsum, J. F., Martin, R. P., Kito, E. & Hess, J. (1956) J. Biol. Chem. **222**, 225–236
Vinay, P., Gougoux, A. & Lemieux, G. (1981) Am. J. Physiol. **241**, F403–F411
Vinuela, E., Salas, M. & Sols, A. (1963) J. Biol. Chem. **238**, 1175–1176
Windmueller, H. G. & Spaeth, A. E. (1981) Am. J. Physiol. **241**, E473–E480

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